Interleukin-22 Promotes Wound Repair in Diabetes by Improving Keratinocyte Pro-Healing Functions

Simona Avitabile[1], Teresa Odorisio[2], Stefania Madonna[1], Stefanie Eyerich[3], Liliana Guerra[4], Kilian Eyerich[5], Giovanna Zambruno[4], Andrea Cavani[1] and Francesca Cianfarani[4]

Impaired re-epithelialization, imbalanced expression of cytokines and growth factors, and vascular disease contribute to healing impairment in diabetes. IL-22, a pro-inflammatory cytokine mediating a cross-talk between immune system and epithelial cells, has been shown to have a role in repair processes. In this study we aimed to investigate IL-22 regenerative potential in the poor healing context of diabetic wounds. By using streptozotocin-induced diabetic mice, we demonstrated that IL-22 wound treatment significantly accelerated the healing process, by promoting re-epithelialization, granulation tissue formation, and vascularization. Improved re-epithelialization was associated with increased keratinocyte proliferation and signal transducer and activator of transcription 3 (STAT3) activation. We showed that endogenous IL-22 content was reduced at both mRNA and protein level during the inflammatory phase of diabetic wounds, with fewer IL-22-positive cells infiltrating the granulation tissue. We demonstrated that IL-22 treatment promoted proliferation and injury repair of hyperglycemic keratinocytes and induced activation of STAT3 and extracellular signal–regulated kinase transduction pathways in keratinocytes grown in hyperglycemic condition or isolated from diabetic patients. Finally, we demonstrated that IL-22 treatment was able to inhibit diabetic keratinocyte differentiation while promoting vascular endothelial growth factor release. Our data indicate a pro-healing role of IL-22 in diabetic wounds, suggesting a therapeutic potential for this cytokine in diabetic ulcer management.

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INTRODUCTION

Chronic diabetic ulcers fail to proceed through biological events characterizing acute repair. Re-epithelialization, new vessel formation, growth factor production, and immune responses are among those functions altered in diabetic ulcers (Loots *et al.*, 1998; Werner and Grose, 2003; Pukstad *et al.*, 2010).

IL-22 is a cytokine involved in host defense at epithelial surfaces and in modulation of tissue responses during inflammatory conditions, including psoriasis and rheumatoid arthritis (Ikeuchi *et al.*, 2005; Zheng, 2007; Wolk *et al.*, 2009). IL-22 is produced by different cell types of innate and adaptive immunity (Trifari *et al.*, 2009; Witte *et al.*, 2010), but the expression of IL-22 receptor complex, IL-22R1/IL-10R2, is mainly confined to epithelial cells of the skin, pancreas, intestine, liver, and lung (Wolk *et al.*, 2004). Binding of IL-22 to its receptor activates the signal transducer and activator of transcription (STAT) pathway, in particular STAT3, and the mitogen-activating peptide kinase pathway (Lejeune *et al.*, 2002).

An important role of IL-22 in repair processes has been emerging in the last decade. IL-22 has been described as involved in liver, colon, thymus, and airway epithelial regeneration (Pickert *et al.*, 2009; Ren *et al.*, 2010; Pociask *et al.*, 2013). In the epidermis, IL-22 induces hyperplasia, inhibiting keratinocyte differentiation, while promoting migration and pro-inflammatory gene expression (Boniface *et al.*, 2005). IL-22$^{-/-}$ mice display delayed cutaneous wound healing (McGee *et al.*, 2013). A beneficial effect of IL-22 has been described in metabolic disorders of diabetic mice, where IL-22 improves insulin sensitivity, preserves gut mucosal barrier, and decreases chronic inflammation (Wang *et al.*, 2014). On the basis of this evidence, we evaluated the effects of IL-22 administration to diabetic wounds and specifically investigated the response of diabetic keratinocytes to IL-22 treatment.

[1]*Laboratory of Experimental Immunology, Istituto Dermopatico dell'Immacolata IRCCS, Rome, Italy;* [2]*Laboratory of Biochemistry, Istituto Dermopatico dell'Immacolata IRCCS, Rome, Italy;* [3]*ZAUM - Center of Allergy and Environment, Technische Universität and Helmholtz Center, Munich, Germany;* [4]*Laboratory of Molecular and Cell Biology, Istituto Dermopatico dell'Immacolata IRCCS, Rome, Italy and* [5]*Department of Dermatology and Allergy, Technische Universität, Munich, Germany*

Correspondence: Andrea Cavani, Laboratory of Experimental Immunology, Istituto Dermopatico dell'Immacolata IRCCS, Via dei Monti di Creta 104, Rome I-00167, Italy or Francesca Cianfarani, Laboratory of Molecular and Cellular Biology, Istituto Dermopatico dell'Immacolata IRCCS, Via dei Monti di Creta 104, Rome I-00167, Italy. E-mail: a.cavani@idi.it or f.cianfarani@idi.it

Abbreviations: ERK, extracellular signal–regulated kinase; INV, involucrin; KRT1, keratin 1; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor

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RESULTS

IL-22 topical treatment accelerates diabetic wound closure by improving re-epithelialization

To evaluate the effect of IL-22 in diabetic wound healing, wounds of diabetic and nondiabetic mice were treated with recombinant mouse IL-22 or with saline control solution. Wound area analysis indicated a significant reduction in IL-22-treated diabetic wounds when compared with saline-treated diabetic controls, starting from day 4 and until day 13 after induction (Figures 1a and b) ($P<0.05$). No significant difference was observed at any time point between nondiabetic and IL-22-treated diabetic mice. All IL-22-treated diabetic wounds healed by day 19 as nondiabetic controls. In contrast, 60% of saline-treated diabetic wounds were not jet healed at that time point (Figure 1b). Cytokine treatment in nondiabetic wounds revealed no significant healing improvement with respect to nondiabetic saline-treated controls (not shown).

To further analyze the effects of IL-22 in diabetic wound healing, histological, and immunohistochemical analyses were performed on 7-day wound biopsies. Re-epithelialization, measured as the percentage of distance covered by the epidermis between the wound edges (Semenova *et al.*, 2008), was significantly increased in IL-22-treated diabetic wounds as compared with diabetic controls ($57.7\pm4.3\%$ in diabetic+IL-22 versus $45.5\pm3.1\%$ in diabetic+saline, $P<0.05$) (Figures 1c and e). Wound contraction, measured as the distance between wound edges, revealed a slight (not significant) increase in IL-22-treated diabetic wounds (Figure 1f). Moreover, immunohistochemistry for the proliferation marker Ki67 revealed a significantly higher percentage of proliferating keratinocytes in the epidermis of IL-22-treated diabetic wounds ($54.46\pm3.04\%$ in diabetic +IL-22 versus $41.7\pm2.2\%$ in diabetic+saline, $P<0.05$) (Figures 1d and g).

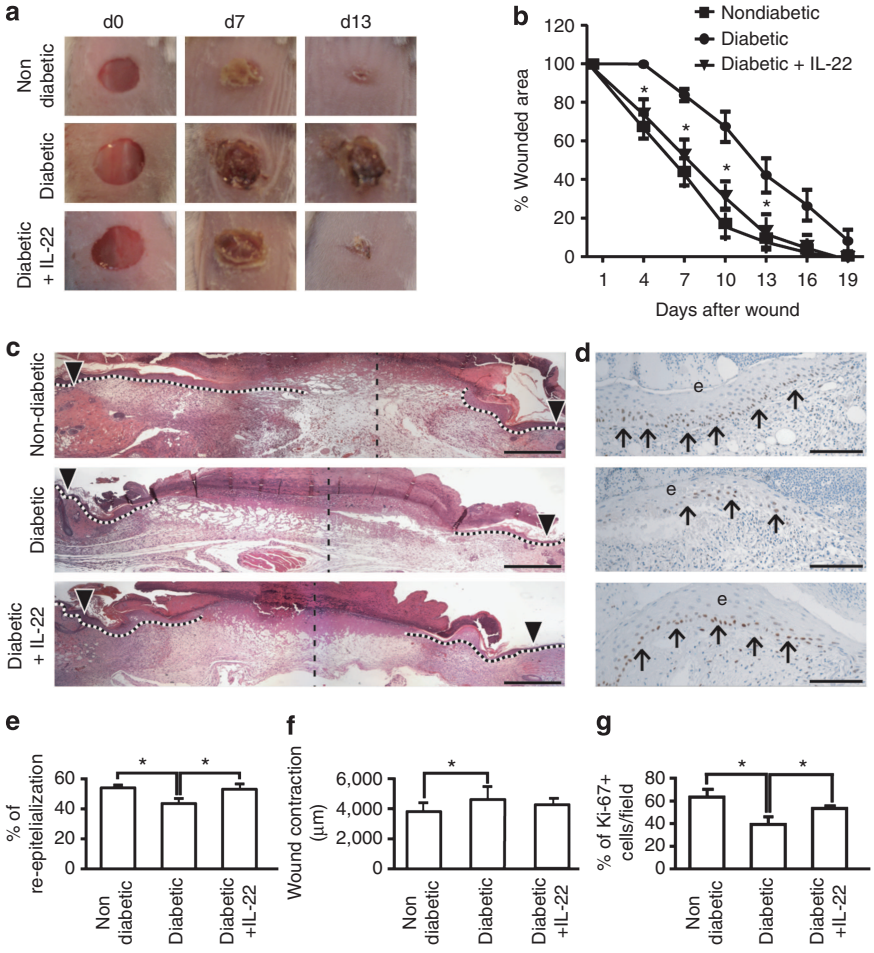

Figure 1. Wound-healing analysis in IL-22-treated diabetic wounds. (**a**) Representative pictures of IL-22- or saline-treated diabetic and nondiabetic wounds at days 0, 7, and 13 after injury. (**b**) Wound-closure analysis of IL-22-treated diabetic (triangles), saline-treated diabetic (circles), and untreated nondiabetic wound areas (squares), ($n=12$ per group). *$P<0.05$. (**c**) Composite pictures of 7-day hematoxilyn and eosin-stained central wound sections of nondiabetic, saline-, and IL-22-treated diabetic wounds. Epithelial tongues (dashed lines) and wound edges (arrows), identified by the most proximal hair follicle, are highlighted. Vertical lines indicate picture arrangement. Bars = 400 µm. (**d**) Ki-67 immunostaining showing proliferating keratinocytes (arrows) in the epidermis (**e**). Bars = 200 µm. (**e**–**g**) Quantitative analysis of re-epithelialization (percentage of distance covered by epidermis) (**e**), contraction (distance between wound margins) (**f**), proliferation (percentage of Ki67-positive keratinocytes) (**g**). ($n=8$–11 per group), *$P<0.05$.

IL-22 administration to diabetic wounds associates with improved granulation tissue formation, maturation, and vascularization

Granulation tissue formation was analyzed by staining central wound sections with Masson's trichrome. Results showed that the granulation tissue area was significantly larger in IL-22-treated wounds compared with saline-treated controls ($1.17 \pm 0.22\ mm^2$ versus $0.55 \pm 0.08\ mm^2$, $P<0.05$) (Figures 2a and c). Moreover, IL-22-treated diabetic wounds also manifested a more mature granulation tissue, largely filled with collagen (green staining) and invading cells, with respect to saline-treated diabetic specimens, almost exclusively filled with fibrin (red staining) and poorly cellularized. Immunohistochemical analysis with an anti-PECAM/CD31 (platelet endothelial cell adhesion molecule-1) antibody revealed significantly increased vascular density in IL-22-treated diabetic wounds (38.5 ± 2.1 vessels per mm^2 in diabetic +IL-22 versus 27.2 ± 3.02 in diabetic controls, $P<0.05$) (Figures 2b and d). All parameters analyzed were similar in IL-22-treated diabetic wounds and nondiabetic untreated controls.

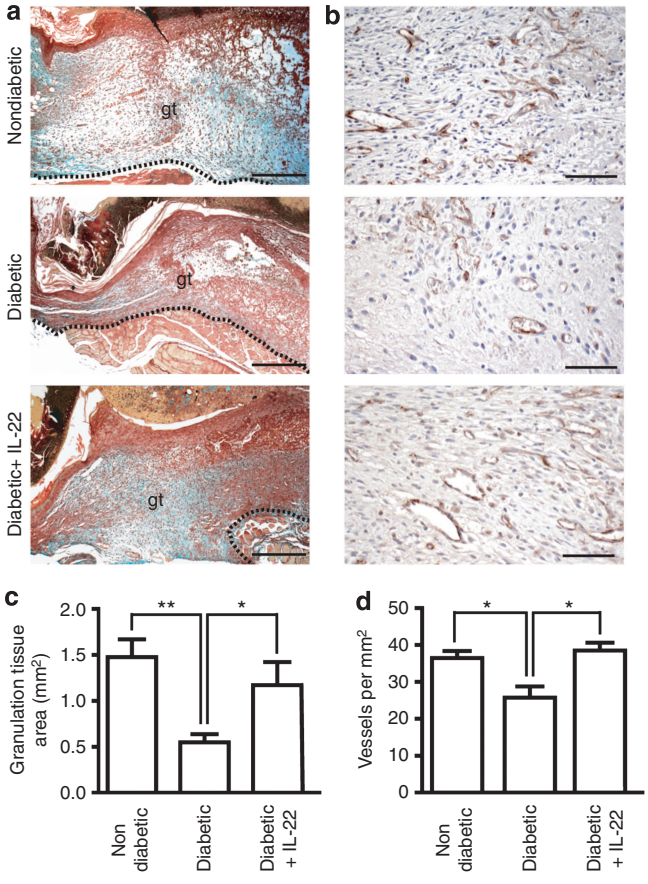

Figure 2. IL-22 effects in diabetic wounds. (**a** and **b**) Seven-day central sections from nondiabetic, saline-treated, and IL-22-treated diabetic wounds. (**a**) Masson's trichrome staining for evaluation granulation tissue (gt) extension and composition. Dashed lines mark the lower boundary between granulation tissue and subcutaneous panniculum carnosum. Bars = 400 μm. (**b**) Anti-platelet endothelial cell adhesion molecule-1 (PECAM/CD31) immunostaining showing blood vessel distribution into granulation tissue. Bars = 100 μm. (**c** and **d**) Quantitative analysis of granulation tissue area (**c**), vessel density (**d**), $n = 8$–11 per group. $^*P<0.05$; $^{**}P<0.01$.

IL-22 expression is reduced in diabetic wounds

To evaluate whether the perturbed diabetic wound-healing process could correlate with altered IL-22 production, IL-22 was analyzed by real-time reverse transcriptase–PCR in RNA extracted by wound biopsies collected at different time points after injury (Figure 3a). In both diabetic and nondiabetic wounds, IL-22 mRNA, undetectable in unwounded skin, was upregulated at day 1 after injury, reaching highest levels at day 3 and then decreasing until day 13. However, in diabetic mice, IL-22 mRNA levels were significantly lower compared with nondiabetic controls at 1, 3, and 7 days (day 1: 1 in diabetic mice, reference value, versus 6.82 ± 2.55 in controls; day 3: 5.27 ± 0.79 versus 13.53 ± 1.2; day 7: 2.01 ± 1.41 versus 5.8 ± 2.01, $P<0.05$). At day 13, IL-22 mRNA levels were similar in both conditions (Figure 3a).

ELISA analysis confirmed that IL-22 was undetectable in nondiabetic and diabetic unwounded skin, and that IL-22 induction reached the maximal level at day 3 after injury, when cytokine content was significantly lower in diabetic wounds (40.92 ± 8.52 versus $67.63 \pm 9.6\ pg\ ml^{-1}$, $P<0.05$) (Figure 3b).

Immunohistochemistry with an anti-IL-22 antibody showed that the IL-22-positive cell number was reduced in day-3 diabetic wounds with respect to nondiabetic controls (307.63 ± 64.11 versus 483.79 ± 37.00 cells per mm^2, $P<0.05$) (Figures 3c and d).

IL-22 accelerates *in-vitro* wound healing of hyperglycemic keratinocytes

To specifically investigate IL-22 activity on diabetic keratinocytes, we performed an *in vitro* injury assay on monolayers of normal human keratinocytes grown in normoglycemic (Norm), hyperglycemic (D-glu), or osmolarity control (L-glu) culture conditions, treated or not with IL-22 (Figure 4a). The absence of toxic effects on cell viability was verified with the LDH (lactate dehydrogenase activity) assay on keratinocytes treated with 50 mM of D- and L-glucose (data not shown). As expected, 16 hours after injury, D-glu-treated keratinocytes displayed significantly delayed closure with respect to Norm or L-glu-treated cells ($87.2\% \pm 0.2$ of initial wounded area in D-glu, $52.2\% \pm 1.8$ in Norm and 55.3 ± 1.3 in L-glu). IL-22 treatment promoted repair in all culture conditions ($3.5\% \pm 0.4$ of initial wounded area in Norm, $2.4\% \pm 0.7$ in L-glu and $9.2\% \pm 0.9$ in D-glu, $P<0.05$) (Figure 4b).

Keratinocyte proliferation was analyzed by BrdU incorporation analysis. Reduced basal proliferation of D-glu-treated keratinocytes was observed (0.6 ± 0.19 in D-glu versus 1 ± 0.11 in Norm, reference value). After 24 hours of stimulation, IL-22 significantly induced proliferation in all culture conditions, recovering the proliferative defect of D-glu-treated cells with respect to controls (1.3 ± 0.02 in Norm, 1.18 ± 0.07 in L-glu and 1.14 ± 0.2 in D-glu, $P<0.05$).

These data indicate that IL-22 efficiently induced migration and proliferation in both normoglycemic and hyperglycemic conditions, recovering the defective behavior of diabetic keratinocytes.

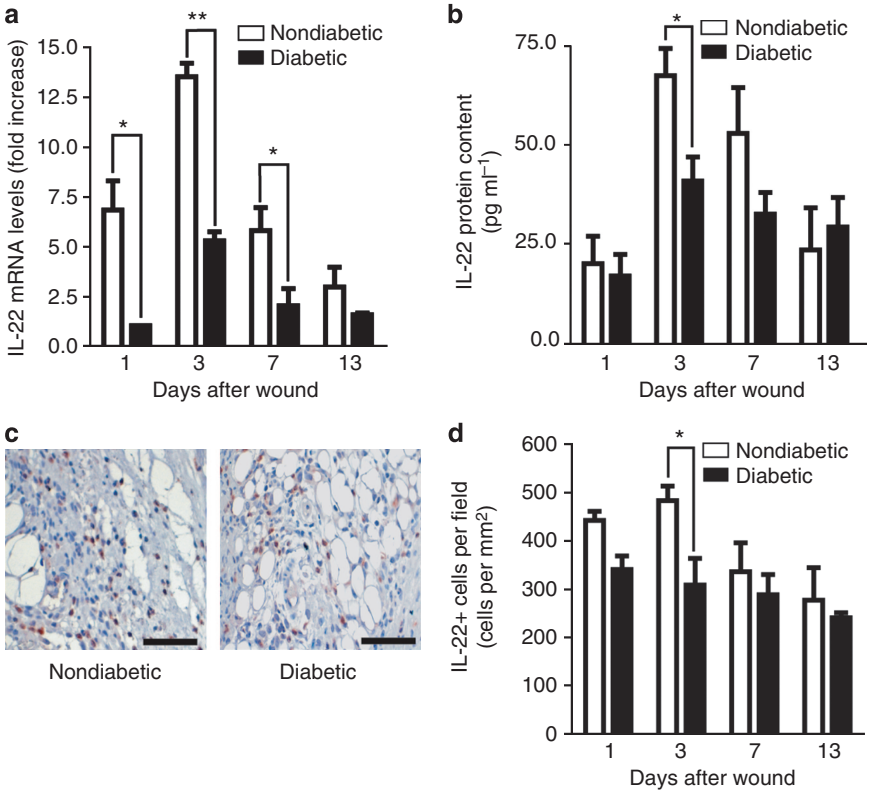

Figure 3. IL-22 expression in diabetic and nondiabetic wounds. (**a**) Real-time PCR analysis of mouse IL-22 mRNA at different time points after wound induction in diabetic and nondiabetic mice. Results are expressed as relative fold induction versus IL-22 expression at day 1 in diabetic mice ($n=3$; $^*P<0.05$; $^{**}P<0.01$). (**b**) ELISA analysis for IL-22 contentin protein extracts from nondiabetic and diabetic wounds at different time points ($n=3$; $^*P<0.05$). (**c**) Immunohistochemistry with anti-mouse IL-22 antibody on day-3 sections of nondiabetic and diabetic wounds showing IL-22-positive cells infiltrating granulation tissue. Bars = 100 μm. (**d**) Quantification of IL-22-positive cell density on wound tissue sections taken at different time points after injury in nondiabetic and diabetic mice ($n=4$; $^*P<0.05$).

IL-22 induces STAT3 and ERK1/2 phosphorylation in diabetic keratinocytes

To verify whether IL-22 treatment was able to activate the canonical STAT3 and extracellular signal–regulated kinase (ERK) intracellular signaling in diabetic keratinocytes, western blot analysis was performed in IL-22-treated normoglycemic and hyperglycemic keratinocytes. IL-22 induced STAT3 phosphorylation in all culture conditions analyzed with a similar fold induction (2.8 ± 0.2 for Norm; 3.0 ± 0.3 for L-glu; 3.6 ± 0.1 for D-glu, $P<0.05$) (Figure 5a). Immunohistochemistry with an anti-phospho STAT3 antibody on murine diabetic wounds revealed that IL-22 was able to activate STAT3 in a significantly higher percentage of proliferating keratinocytes compared with saline-treated controls ($48.59\%\pm8.3$ versus $27.01\%\pm2.6$, $P<0.01$) (Figure 5e). IL-22 also induced ERK1/2 phosphorylation in hyperglycemic cultured keratinocytes, with a fold increase similar to those exhibited by normoglycemic and L-glucose-treated cells (2.5 ± 0.7, 2.4 ± 0.1, and 2.3 ± 0.2, respectively, $P<0.05$) (Figure 5b).

In parallel, signaling activation was analyzed on primary keratinocytes from diabetic and nondiabetic individuals. STAT3 phosphorylation was significantly induced by IL-22 in both specimens, but induction observed in diabetic cells was significantly higher as compared with nondiabetic ones (3.6 ± 0.3 versus 1.9 ± 0.1, $P<0.05$) (Figure 5c). IL-22 triggered ERK1/2 phosphorylation to a similar extent in diabetic and nondiabetic keratinocytes (2.4 ± 0.1 versus 2.5 ± 0.1, $P<0.05$) (Figure 5d). These results demonstrated that IL-22 is able to drive STAT3 and ERK activation in diabetic keratinocytes.

IL-22 modulates Keratin 1 and Involucrin expression and VEGF secretion in diabetic keratinocytes

IL-22 has an anti-differentiating role in keratinocytes also by inhibiting the expression of the differentiation marker keratin-1 (KRT1), in a STAT3-dependent manner (Sestito *et al.*, 2011). To analyze whether IL-22 was able to counteract excessive differentiation that afflicts diabetic keratinocytes (Sprachikov *et al.*, 2001), we evaluated the expression of the differentiation markers KRT1 and involucrin (INV). IL-22 was added in the culture medium of keratinocytes of diabetic and nondiabetic individuals induced to differentiate by 4 days over confluence growing.

In sub-confluent cells (days 0), KRT1 and INV expression levels were significantly higher in diabetic keratinocytes (fold increase: 1.9 ± 0.1 for KRT1 and 1.3 ± 0.05 for INV in diabetic versus nondiabetic). After differentiation induction, KRT1 and INV were significantly induced in both cell types, in basal

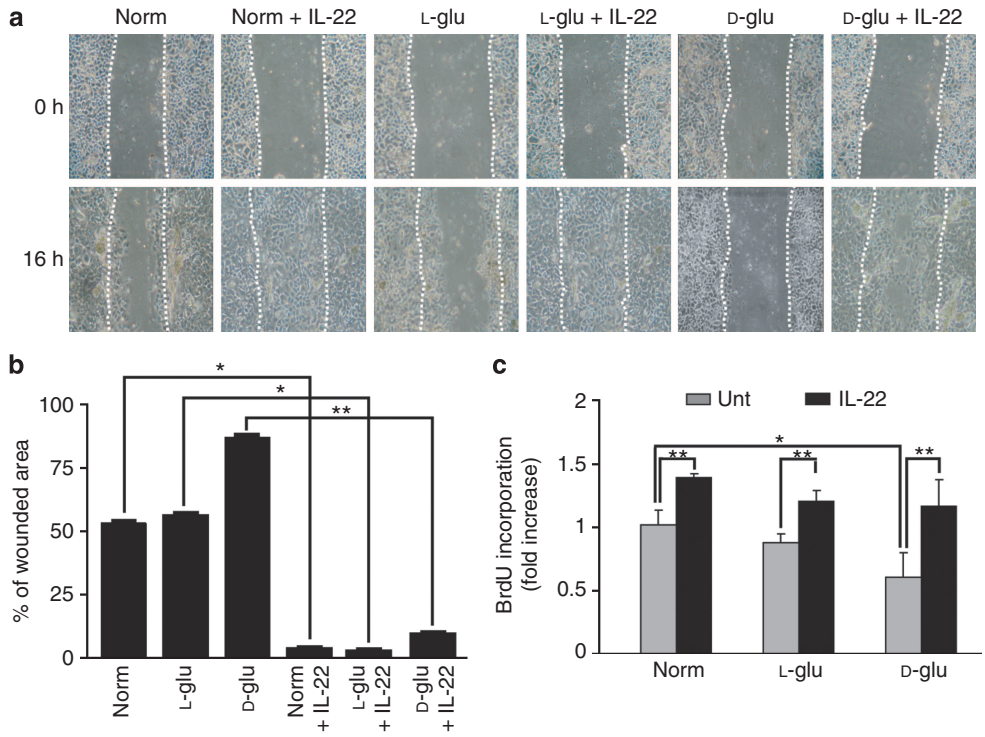

Figure 4. IL-22 promotes wound healing and stimulates proliferation in normoglycemic and hyperglycemic keratinocytes. Scratch assays (**a** and **b**) and BrdU incorporation analysis (**c**) on human keratinocytes grown in normoglycemic (Norm) and hyperglycemic (D-glucose (D-glu) and L-glucose (L-glu) as osmolarity control) conditions, treated or not with 50 ng ml^{-1} of human recombinant IL-22. (**a**) Microscopic images were taken immediately after (0 hour) and 16 hours after (16 hours) wound induction on confluent cell layers, and cell-free area was measured (**b**). (**c**) Proliferation rate was measured 24 hours after IL-22 treatment and expressed as fold increase versus normoglycemic untreated keratinocytes, to which a value of 1 was given. Results were obtained from two independent experiments, each performed on three different keratinocyte strains. (*$P<0.05$; **$P<0.01$).

condition (3.0 ± 0.2 in nondiabetic, 9.0 ± 0.3 in diabetic, for KRT1; 1.7 ± 0.1 in nondiabetic, 2.3 ± 0.03 in diabetic, for INV, fold increase versus nondiabetic basal level, $P<0.05$). IL-22 treatment was able to exert its anti-differentiating role by restoring KRT1 and INV levels in both nondiabetic and diabetic keratinocytes (fold increase: 0.83 ± 0.2 and 1.83 ± 0.2, respectively, for KRT1; 1.2 ± 0.08 and 1.29 ± 0.1 for INV, $P<0.05$) (Figures 6a and b).

Having found increased vessel density in IL-22-treated diabetic wounds, we investigated whether IL-22 could promote pro-angiogenic factor vascular endothelial growth factor (VEGF) secretion in keratinocytes. VEGF release was analyzed by ELISA assay in supernatants of keratinocytes from diabetic patients and nondiabetic controls, treated or not with IL-22. VEGF secretion was reduced in diabetic keratinocytes compared with controls, in basal condition (0.5 ± 0.09 fold changes). IL-22 treatment significantly stimulated VEGF production in both diabetic and nondiabetic cells (1.15 ± 0.28 in diabetic; 2.3 ± 0.05 in nondiabetic, over untreated nondiabetic, $P<0.05$) (Figure 6c).

DISCUSSION

During cutaneous wound healing, early inflammatory responses occur, thus preventing infections and enhancing repair via the release of cytokine signaling between immune and other skin cell populations. Growing evidences point to IL-22 as a cytokine involved in repair events. IL-22 pro-healing functions were described in studies on keratinocytes (Boniface *et al.*, 2005; Eyerich *et al.*, 2009) and on different models of epithelial regeneration (Pickert *et al.*, 2009; Ren *et al.*, 2010; Dudakov *et al.*, 2012; Pociask *et al.*, 2013); however, a description of the biological effects induced by IL-22 in cutaneous wound healing is still lacking. In this study, we asked whether IL-22 could contribute to restore wound-healing processes altered in diabetic skin. We used streptozotocin-induced type I diabetic mice, manifesting wound-healing impairment, and used to test molecules with therapeutic potential for diabetic wounds (Graiani et al., 2004; Fadini *et al.*, 2010; Lim et al., 2015). We found that IL-22 treatment significantly accelerated diabetic wound closure, improving it to the levels of nondiabetic wounds. In particular, IL-22 promoted diabetic wound re-epithelialization, also by increasing the number of proliferating keratinocytes in the epithelial tongue of diabetic lesions. *In-vitro* analysis revealed that IL-22 is able to directly stimulate migration and proliferation in hyperglycemic keratinocytes. Improved re-epithelialization might also be due to inhibition of terminal keratinocyte differentiation, as this was shown to be the mechanistic basis of the observed IL-22-induced epidermal hyperplasia in a model of reconstituted human

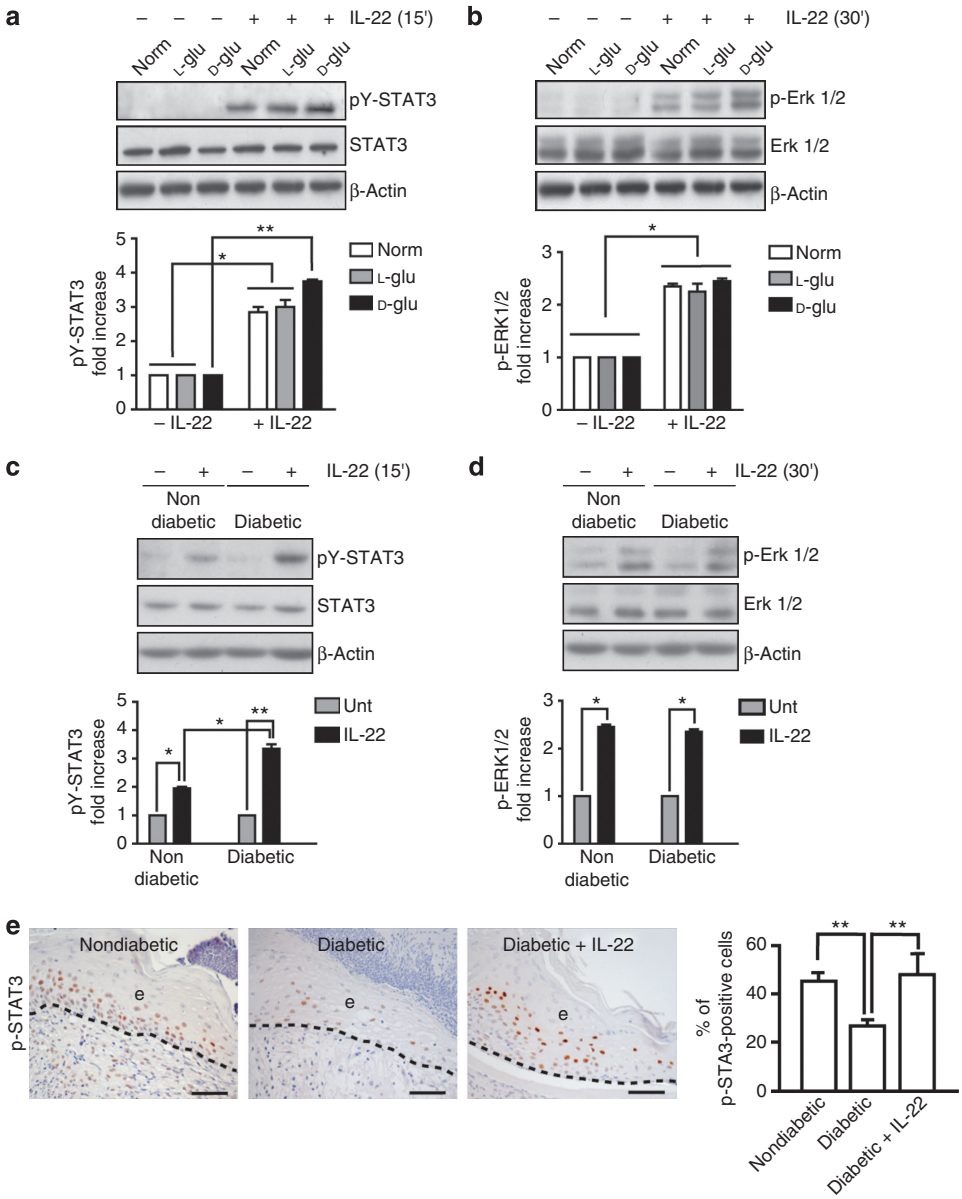

Figure 5. Signal transducer and activator of transcription 3 (STAT3) and extracellular signal–regulated kinase (ERK) signaling analysis in IL-22-treated diabetic keratinocytes. Western blotting and relative quantifications on protein extracts from keratinocytes grown in normoglycemic (Norm) and hyperglycemic conditions (L-glucose (L-glu) and D-glucose (D-glu)) (**a** and **b**) or from keratinocytes from diabetic and nondiabetic individuals (**c** and **d**), treated or not with IL-22. STAT3 (**a** and **c**) and ERK1/2 (**b** and **d**) phosphorylation levels after 15′ and 30′ (respectively) of stimulation with 50 ng ml^{-1} of IL-22. $n=3$. $^{*}P<0.05$; $^{**}P<0.01$. Composite images (**c** and **d**) are related to different parts of the same gel. (**e**) *P*-STAT3 immunostaining on 7-day nondiabetic and diabetic mouse wounds treated or not with IL-22, showing immunopositive keratinocytes in the epidermis (**e**) (dashed lines show dermal–epidermal junction) and relative quantification of the percentage of *P*-STAT3-positive cells. $n=8$, $^{*}P<0.05$; $^{**}P<0.01$. Bars = 100 μm.

epidermis (Boniface *et al.*, 2005). Keratinocyte differentiation and their consequent capacity to proliferate are reflected by keratin expression pattern. In particular, KRT1 is associated with differentiating cells of suprabasal epidermal layers (Koster, Roop, 2007) and excessive differentiation is a typical feature of diabetic keratinocytes (Sprachikov *et al.*, 2001). IL-22 was previously shown to downregulate KRT1 expression via STAT3 activation (Sestito *et al.*, 2011). In line with these observations, we found that KRT1 expression is higher in diabetic keratinocytes than in nondiabetic control cells, and that IL-22 treatment sensibly reduces KRT1 levels also in the more differentiated diabetic keratinocytes. Analogous results were obtained analyzing IL-22 effects on INV expression.

Confirming previous reports about a central role for STAT3 as the mechanistic basis for skin remodeling and keratinocyte migration (Sano *et al.*, 1999; Kira *et al.*, 2002), we show that IL-22 significantly induces STAT3 phosphorylation both *in vitro* in hyperglycemic and diabetic patients keratinocytes and *in vivo* in keratinocytes of diabetic murine wounds

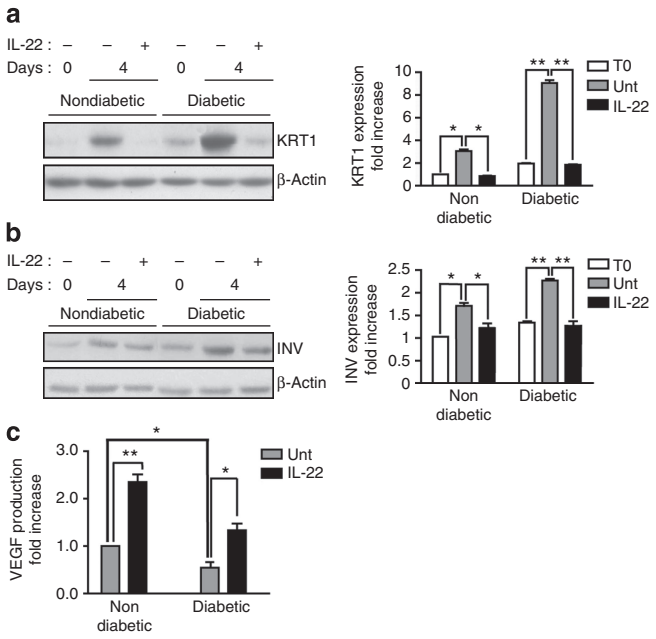

Figure 6. Keratin 1 (KRT1) and involucrin (INV) expression and vascular endothelial growth factor (VEGF) production in IL-22-treated diabetic keratinocytes. (**a**) Western blot analysis and relative densitometric quantification of KRT1 (**a**) and INV (**b**) levels on protein lysates from diabetic and nondiabetic keratinocyte strains treated or not (Unt) with 50 ng ml^{-1} of IL-22 for 48 hours. Analysis was performed on keratinocytes before and after terminal differentiation achieved by growing cells for 4 days after confluence in the presence or absence of IL-22 ($n=3$; $^{*}P<0.05$; $^{**}P<0.01$). Western blotting composite images are related to different parts of a same gel. (**c**) ELISA assay measuring VEGF content in supernatants of human diabetic and nondiabetic keratinocytes cultured for 24 hours in the presence or the absence of IL-22 ($n=3$; $^{*}P<0.05$).

treated with IL-22. Besides STAT3, IL-22 also induces the activation of the keratinocyte pro-proliferative and pro-survival ERK1/2 pathway (Pastore *et al.*, 2005; Zhang et al 2012), in both hyperglycemic and diabetic patient keratinocytes.

IL-22-treated diabetic wounds also exhibited granulation tissue formation to a level comparable to normal wounds. In the skin, only keratinocytes and, to a minor extent, dermal fibroblasts express IL-10R/IL-22R, thus being IL-22 targets (Wolk *et al.*, 2009). Thus, the improvement of granulation tissue extension in diabetic wounds treated with IL-22 could be attributed to a direct action on dermal fibroblasts, with a consequent stimulation of extracellular matrix protein deposition. In parallel, we can also hypothesize an indirect stimulation of fibroblasts by growth factors released by IL-22-activated keratinocytes.

Importantly, we found that IL-22 administration also associated with increased vascular density in diabetic wounds, where vessel density reached values similar to those of nondiabetic controls. This effect is highly relevant for the rescue of diabetic wound repair impairment, as vascular complications are regarded as the leading cause of defective healing in diabetes. VEGF expression is reduced in diabetic ulcers (Frank *et al.*, 1995) and VEGF treatment accelerates diabetic healing by promoting angiogenesis (Romano Di Peppe, Mangoni, 2002; Galiano *et al.*, 2004). The observed angiogenic effect cannot be directly induced by administered IL-22, as endothelial cells lack IL-22 receptor (Wolk *et al.*, 2009). During wound healing, actively proliferating keratinocytes are an important source of VEGF that stimulates proliferation and tropism of endothelial cells into the granulation tissue (Brown *et al.*, 1992). We found that IL-22 promotes VEGF secretion in diabetic keratinocytes. Moreover, STAT3 is a transcription activator for VEGF, whose expression is silenced following STAT3 targeting (Xu *et al.*, 2005). Therefore, STAT3 activation mediated by IL-22 may modulate VEGF secretion in both nondiabetic and diabetic keratinocytes, thus indirectly supporting the increased blood vessel density observed in IL-22-treated wounds. The effects of IL-22 in the diabetic wound might be of high clinical relevance, as we observed significantly reduced cytokine levels at early time points, reflected by the decreased number of IL-22-positive cells at the wound site. Efficient immune responses are essential for tissue repair outcome (Schaffer, Barbul, 1998). CD4^{+} T lymphocyte–impaired recruitment, functional reduction, and lower cytokine secretion capacity have been described in human chronic lesions (Loots *et al.*, 1998; Toulon *et al.*, 2009). We showed how IL-22 expression was undetectable in both normal and diabetic unwounded skin. After wound induction, we observed a time-regulated increase in IL-22, significantly reduced in diabetic condition. Proliferation of resident skin γ/δT cells, among major IL-22 producers, is compromised in diabetic mice (Taylor *et al.*, 2010). However, increased IL-22 circulating levels were described in type II diabetic patients (Guo *et al.*, 2014; Zhao *et al.*, 2014). We can then hypothesize that reduced IL-22 content in diabetic wounds could be determined by a local impaired activation of these cell populations, in addition to altered recruitment of circulating immune cells producing IL-22 into the diabetic wound tissue, a microenvironment not obviously mirroring the systemic situation. Further investigations will be necessary to characterize the immunological profile of these IL-22-positive cells.

In conclusion, our results indicate an innovative role for IL-22 in diabetic wound repair, exerted not only by improving re-epithelialization, granulation tissue deposition, and vascularization but also by modulating keratinocyte differentiation and VEGF production. Globally, our data suggest IL-22 as a potential tool for therapeutic treatment of diabetic wounds.

MATERIALS AND METHODS

Diabetic mice and wound-closure analysis

Animal experiments were approved by the Istituto Dermopatico dell'Immacolata, IDI-IRCCS Animal Care and Use Committee. Six-week-old Balb/c male mice (Harlan Laboratories, San Pietro al Natisone, Italy) were rendered diabetic by a single intraperitoneal injection of streptozotocin (180 mg kg^{-1}) (Sigma-Aldrich, St. Louis, MO). After 1 week, blood glucose levels were measured and mice with glycemia ranging between 285 mg dl^{-1} (minimum level) and 590 mg dl^{-1} (maximum level) were used 3 weeks later for wound experiments (average glycemic levels: 476 mg dl^{-1}). During experimental procedures, diabetic mice were weekly weighted and

monitored for polyuria, polyphagia, polydipsia, and glycemia. Mice manifesting a body weight loss above 20% or a poor motility were excluded. Changes in body weight of the selected diabetic animals were as follows: (average values ± s.d.): 20.62 g ± 1.48 (before treatment); 18.985 g ± 1.27 (1 week after streptozotocin); 18.385 g ± 1.14 (end of the experiment). A 6-mm-diameter full-thickness excisional wound was performed on the dorsum of diabetic and nondiabetic mice. At days 1 and 4 after injury, 1 μg of mouse recombinant IL-22 (R&D Systems, Abingdon, UK) or analogous volume of saline control solution was injected below the crust. Wound margin was traced on a transparency film at days 0, 1, and every third day thereafter until closure. Wound areas were measured by a computer-assisted image analyzer (KS300; Zeiss, Jena, Germany) and the healing rate expressed as the percentage of initial area.

Histological and immunohistochemical analysis

Diabetic mice treated with IL-22 or saline and nondiabetic controls were killed 7 days after wounding and wound biopsies excised, paraformaldehyde fixed, and paraffin embedded. Four-micrometer-thick central wound sections were processed (Failla *et al.*, 2000). Ematoxilin/eosin and Masson's trichrome staining (DakoCytomation, Milan, Italy) were performed to analyze re-epithelialization and granulation tissue formation, respectively. Antibodies used were as follows: anti-PECAM/CD31 (M 20; Santa Cruz Biotechnology, Santa Cruz, CA), anti Ki67 (Novocastra-Leica Microsystems, Milan, Italy), anti-IL-22 (Novus Biologicals, Cambridge, UK) and anti *P*-STAT3 (Cell Signaling, Danvers, MA). Negative controls were obtained by omitting primary antibody. Samples were quantified by computer-assisted image analyzer (Zeiss). Analyses were performed by two blinded operators on six to eight noncontiguous, randomly selected fields/sample.

Real-time reverse transcriptase–PCR

Total RNA was extracted from wound biopsies and converted into cDNA as described (Cianfarani *et al.* 2006). Real-time reverse transcriptase–PCR was performed with SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA) using the following primers: IL-22 forward 5′-TCCGAGGAGTCAGTGCTAAA-3′, reverse 5′-AGAACGTCTTCCAGGGTGAA-3; glyceraldehyde-3-phosphate dehydrogenase forward 5′-GTATGACTCCACTCACGGCAAA-3′, reverse 5′-TTC-CCATTCTCGGCCTTG-3′. PCR products were measured by the ABI PRISM 5700 detection system (Perkin-Elmer, Norwalk, CT) and normalized to glyceraldehyde-3-phosphate dehydrogenase amplification. Quantification was performed by the comparative CT method (Pfaffl, 2001). All determinations were performed in triplicate.

Keratinocyte cultures

Primary cultures of human keratinocytes (Madonna *et al.*, 2008) were obtained from skin biopsies of three type II diabetic patients and three healthy volunteers enrolled in the study after written informed patients consent. The study was performed according to the Declaration of Helsinki Principles with regard to scientific use and approved by the ethical committee of Istituo Dermopatico dell'Immacolata, IDI-IRCCS.

Keratinocytes were grown in a serum-free keratinocyte growth medium (KGM; Clonetics, Walkersville, MD), until 40–60% confluence. For conditions, 50 mM of D-glu and L-glu (Sigma Aldrich) were added as hyperglycemic and osmolarity control condition, respectively. All stimulations with recombinant human IL-22 (R&D Systems) were performed in KBM.

Scratch and BrdU incorporation assays

Normo and hyperglycemic keratinocytes were grown to confluence on type IV collagen (20 μg ml^{-1}) and then scratched with a *P*-200 pipette tip. Cultures were incubated with respective media containing or not human recombinant IL-22. The cell-free area was measured with a computer-assisted image analysis system (Zeiss) and expressed as the percentage of initial wounded area.

Normo and hyperglycemic keratinocytes were plated in 96-well plates (2×10^4 cells per well) and stimulated or not with human recombinant IL-22 for 24 hours. BrdU was added 16 hours before the end of the incubation time and its incorporation analyzed by using the BrdU cell proliferation kit (Roche Applied Sciences, Mannheim, Germany).

Western blot analysis

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors (Roche). Proteins were resolved on a 10% SDS–PAGE, transferred to polyvinylidene difluoride filters, and probed with primary antibodies. Filters were developed using the ECL-plus detection system (Amersham Biosciences, Buckinghamshire, UK) or the SuperSignal West Femto kit (Pierce, Rockford, IL) and probed with phospho-YSTAT3 (Tyr 705) (Cell Signaling), STAT3, *P*-ERK1/2, ERK1/2, β-actin (Santa Cruz Biotechnology), KRT1, and INV, (Covance, Emeryville, CA). Immunoblots were quantified by imaging densitometer (GS-670; Bio-Rad, Hercules, CA).

ELISA assay

Protein extracts from wound biopsies and cultured human keratinocytes were obtained as described (Odorisio *et al.*, 2002). IL-22 and VEGF were, respectively, quantified in wound extracts and keratinocyte supernatants by using IL-22 murine Quantikine and VEGF Quantikine Elisa Kits (R&D Systems).

Statistical analysis

Statistical significance was determined using Student's t-test. Values were expressed as mean ± s.d. P-values <0.05 were considered statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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